

REVIEW

Structure and Function of Sea Urchin Egg Jelly Molecules

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ABSTRACT—Sea urchin egg jelly consists of a fucose sulfate glycoconjugate, a sialoglycoprotein and sperm-activating peptides. The fucose sulfate glycoconjugate isolated from the solubilized jelly layer of *Hemicentrotus pulcherrimus* is composed of several proteins (a sugar-containing core protein, a 258 kDa protein, a 237 kDa protein and a 120 kDa protein); the substance plays a major role in the induction of the acrosome reaction in *Hemicentrotus pulcherrimus* spermatozoa. The protein components of the fucose sulfate glycoconjugate seem to be associated with one another through disulfide bonds. Sixty-three sperm-activating peptides have been isolated from the solubilized jelly layers of fifteen sea urchin species distributed over four taxonomic orders. Sperm-activating peptides stimulate sperm respiration and motility, increase the levels of both cAMP and cGMP in sea urchin sperm cells, induce net H^+ efflux from sea urchin spermatozoa and cause a shift in the mobility of guanylate cyclase, a major sperm plasma protein, on an SDS-polyacrylamide gel. These peptides are specific at the ordinal level and are classified into four groups, i.e., sperm-activating peptide I (SAP-I), sperm-activating peptide II (SAP-II), sperm-activating peptide III (SAP-III) and sperm-activating peptide IV (SAP-IV). SAP-I (Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly) promotes the induction of the acrosome reaction in *Hemicentrotus pulcherrimus* spermatozoa by acting as a specific co-factor of the fucose sulfate glycoconjugate.

INTRODUCTION

Sea urchin eggs are surrounded by a transparent, gelatinous matrix called the jelly layer, through which spermatozoa must pass before reaching the plasma membrane of the egg during fertilization. The jelly layer has been shown to induce the acrosome reaction, which was first described in detail by Dan [1–14]. The jelly layer of sea urchins mainly consists of polysaccharide-protein complexes which can be separated into a fucose sulfate glycoconjugate and a sialoglycoprotein [13, 15–22]. The latter is considered to cause sperm-isoagglutination and the former to be responsible for the induction of the acrosome reaction. The acrosome reaction in spermatozoa is an essential requirement for fertilization of eggs in many animals [23–43]. In sea urchins, the acrosome reaction occurs within seconds after sperma-

tozoa have come into contact with the egg jelly layer [4, 9–14, 43] or the egg surface component, a sperm-binding factor on the vitelline membrane [45].

Considering the large number of acidic groups belonging to the ester-linked sulfate in the fucose sulfate glycoconjugate and carboxyl residues in the bound sialic acids of the sialoglycoprotein, it follows that the pH values within the jelly layer would be lower than the pH of normal sea water [46–47]. At low pH values, the respiration and motility of sea urchin spermatozoa are markedly reduced and their movements in the jelly layer, therefore, are slower than those that prevail in sea water [48–51]. Thus, one can surmise that the egg jelly is provided with a system of controlling motility and respiration, because the spermatozoa need to maintain their activity at the initial high level attained in sea water during their passage through the acidic environment of the jelly layer to reach the egg surface.

It has been known for over 70 years that the jelly layers of certain species of sea urchins, dissolved in sea water, are able to cause a transient activation of sperm motility and sperm agglutination [52]. Since then many investigators have shown that soluble factors associated with sea urchin eggs stimulate the motility and respiration of sea urchin spermatozoa [53–58]. However, such stimulation has only been seen on occasion and has been difficult to reproduce. In 1976, Ohtake demonstrated that the respiration of sea urchin spermatozoa could be reproducibly stimulated by jelly layer factors obtained from the sea urchin *Pseudocentrotus depressus* if the extracellular pH was maintained at acidic values [50–51]. Thereafter, Kopf *et al.* and Hansbrough and Garbers reported that the jelly layer of the sea urchin *Strongylocentrotus purpuratus* contains a substance which elevates the respiration rates and cyclic nucleotide concentrations in *Strongylocentrotus purpuratus* spermatozoa [59–61]. Hansbrough and Garbers named the substance speract which stands for sperm-activating peptide. In 1981, my collaborators and I first purified a substance from the solubilized jelly

layer of the sea urchin *Hemicentrotus pulcherrimus* and demonstrated that the substance is a decapeptide whose structure is Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly and stimulates the respiration and motility of *Hemicentrotus pulcherrimus* spermatozoa [62]. The same peptide has also been purified from the solubilized jelly layer of *Strongylocentrotus purpuratus* [63].

PURIFICATION OF EGG JELLY MOLECULES

Since the jelly layer of sea urchin mainly consists of highly viscous large molecular weight polysaccharide-protein complexes, it is quite difficult to deal with an intact jelly layer using common biochemical methods. In preparation for analysis of the jelly layer, it must first be dissolved, which is accomplished by treating eggs with sea water adjusted to pH 5.0 or 5.5 (Fig. 1). The centrifuged supernatant (solubilized jelly layer) is used for the purification of jelly molecules.

To purify sperm-activating peptides, the solubilized jelly layer is mixed with a 2-fold volume of

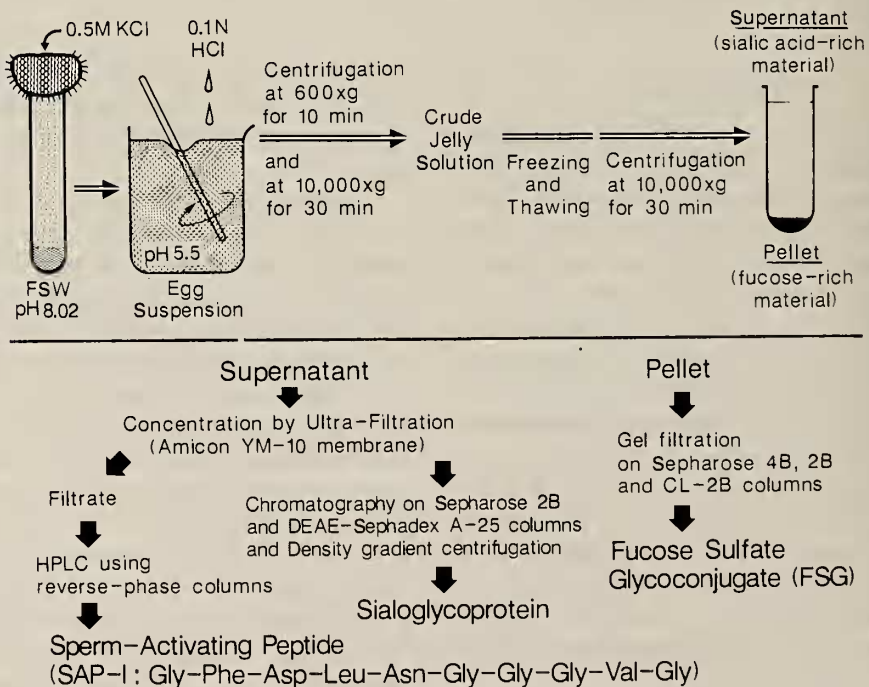


FIG. 1. Schematic drawing of the purification procedure for sea urchin egg jelly molecules.

99% ethanol and then centrifuged. The resulting supernatant is concentrated under reduced pressure at 50°C and lipids are removed by chloroform extraction. The water layer is then lyophilized and the residue, dissolved in deionized and distilled water (DDW), is used for peptide purification. When my collaborators and I first isolated sperm-activating peptides from the solubilized jelly layer of *Hemicentrotus pulcherrimus*, we used sequential chromatographies on Sephadex G-25, DEAE-Sephadex A-25, Sephadex G-15 and Avicell SF thin layer plate [62]. At that time we used about 20 liters of solubilized jelly layer prepared from 5,000 female sea urchins. This procedure, however, was time-consuming and required a large amount of solubilized jelly layer to obtain pure peptides. We then modified the purification procedure and we now use sequential high performance liquid chromatography (HPLC) on a reverse-phase column to purify the peptides [64]. In general, separations are carried out using a combination of the following programs. Program I: Flow rate is 9.9 ml/min, the column (Shimpack C-8 Prep, particle size 5 μ m, 20 $\times$ 250 mm) is equilibrated with 5% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) in DDW and eluted 15 min with equilibration solvent, followed by elution with 60% ACN in TFA in DDW for the next 15 min. Program II: Flow rate is 1.0 ml/min, the column (Unisil C-8, particle size 5 μ m, 4.6 $\times$ 250 mm) is equilibrated with 10% ACN in 0.1% TFA in DDW and eluted for 10 min with equilibration solvent, followed by a linear gradient of ACN from 10% to 50% in 0.1% TFA in DDW over a 50 min time period. Program III: Flow rate is 1.0 ml/min, the same column used in Program II is equilibrated with 5% ACN in 5 mM sodium phosphate (pH 5.7) and eluted for 20 min with the equilibration solvent, followed by a linear gradient of ACN from 5% to 30% in 5 mM sodium phosphate (pH 5.7) over a 40 min time period. By using this modified procedure, we purified three new sperm-activating peptides from solubilized jelly layer obtained from 150 female *Hemicentrotus pulcherrimus* sea urchins.

For purification of the large molecular weight components of the jelly layer, solubilized jelly layer is dialyzed against 0.1 M NaCl and then applied to a Sepharose 2B column equilibrated

with 0.1 M NaCl at 4°C [17–18, 65]. Fucose-containing material is eluted earlier than the material containing sialic acid. To purify a fucose sulfate glycoconjugate, fractions containing fucose are subjected to chromatography on a Sepharose CL-2B column with 7 M urea containing 10 mM HEPES (pH 7.0) [18, 65]. The purified fucose sulfate glycoconjugate which contains one mol sulfate/mol fucose possesses 2.0 times the amount of protein to fucose by weight. When further purification is needed, a fucose sulfate glycoconjugate containing-fraction obtained from Sepharose CL-2B gel filtration is subjected to HPLC on a TSK G-6000 PW column equilibrated with 0.1 M sodium phosphate (pH 6.8) containing 0.1% SDS.

Fractions containing sialic acid obtained from chromatography of solubilized jelly layer on a Sepharose 2B column are pooled and then dialyzed against 10 mM sodium acetate (pH 5.0) containing 0.1 M NaCl and chromatographed on a DEAE-Sephadex A-25 column with a linear gradient of NaCl from 0.1 M to 1.2 M in sodium acetate (pH 5.0) [65]. Fraction containing sialic acid are pooled and dialyzed against DDW. Solid guanidine hydrochloride, CsCl and 1 M sodium acetate (pH 5.0) are added to the dialysate at final concentrations of 4 M, 0.47 g/ml and 10 mM, respectively. The solution is centrifuged at 100,000 $\times g$ for 48 hr in a Hitachi RPS 50 rotor at 4°C. A sialoglycoprotein is obtained from the clear, bottom fraction with a density of 1.47 g/ml. The sialoglycoprotein consists of sialic acid (90%, w/w) and protein (10%, w/w).

It should be noted that in the case of *Hemicentrotus pulcherrimus*, the fucose-containing material becomes insoluble in a 1 M NaCl solution upon freeze-thawing. About 80% of the fucose in the original solubilized jelly layer is recovered in the precipitate fraction by centrifugation at 10,000 $\times g$ for 30 min (Fig. 1) [65]. The precipitate thus obtained dissolves easily in DDW but not in artificial sea water (ASW) or even in 4 M guanidine hydrochloride solution. The supernatant fraction contains about 76% of the sialic acid present in the original solubilized jelly layer. Thus, we sometimes use the precipitate or the supernatant fraction for the purification of a fucose sulfate glycoconjugate or a sialoglycoprotein. However, this

works only with solubilized jelly layer prepared from *Hemicentrotus pulcherrimus* eggs. The solubilized jelly layer obtained from *Anthocidaris cras-*

sispina, *Strongylocentrotus nudus*, *Pseudocentrotus depressus* or *Clypeaster japonicus* does not precipitate upon freeze-thawing [66].

TABLE 1. Sperm-activating peptides from the egg jelly of various species of sea urchins*

Subclass Regularia	
Order Diadematoidea	
Suborder Diadema	
Family Diadematoidea	
<i>Diadema setosum</i>	
GCPWGGAVC	(SAP-IV, Mw 847)
Order Arbacioida	
Suborder Phymosomatina	
Family Phymosomatidae	
<i>Glyptocidaris crenularis</i>	
SAKLCPGGNCV	(Ser, Ala-SAP-IIB, Mw 1048)
KLCPGGNCV	(SAP-IIB, Mw 890)
LCPGGNCV	(Des-Lys ¹ -SAP-IIB, Mw 762)
SFKLCPGGQCV	(ser, Phe-[Gln ⁷]-SAP-IIB, Mw 1138)
KLCPGGQCV	([Gln ⁷]-SAP-IIB, Mw 904)
LCPGGQCV	(Des-Lys ¹ -[Gln ⁷]-SAP-IIB, Mw 776)
Suborder Arbacina	
Family Arbaciidae	
CVTGAPGCVGGGRL-NH ₂	(SAP-IIA, Mw 1245)
Order Echinoida	
Suborder Temnopleurina	
Family Toxopneustidae	
<i>Lytechinus pictus</i>	
GFDLTGGGVQ	([Thr ⁵ , Gln ¹⁰]-SAP-I, Mw 950)
FDLTGGGVQ	(Des-Gly ¹ -[Thr ⁵ , Gln ¹⁰]-SAP-I, Mw 893)
<i>Tripneustes gratilla</i>	
GFDLNGGGVG	(SAP-I, 892)
GFNLNGGGVG	(Asn ³]-SAP-I, Mw 891)
GFSIGGGVG	([Ser ³ , Ile ⁴ , Gly ⁵]-SAP-I, Mw 807)
GFDLGGGGVG	([Gly ⁵]-SAP-I, Mw 835)
GFSLGGGGVG	([Ser ³ , Gly ⁵]-SAP-I, Mw 807)
GFGLGGGGVG	([Gly ^{3,5}]-SAP-I, Mw 777)
G(Br-F)NLNGGGVG	([Br-Phe ² , Asn ³]-SAP-I, Mw 971)
G(Br-F)DLNGGGVG	([Br-Phe ²]-SAP-I, Mw 972)
<i>Pseudoboletia maculata</i>	
GFALDGVN	(Des-Gly ^{6,7} -[Ala ³ , Asp ⁵ , Asn ¹⁰]-SAP-I, Mw 792)
GFALDGVG	(Des-Gly ^{6,7} -[Ala ³ , Asp ⁵]-SAP-I, Mw 735)
<i>Pseudocentrotus depressus</i>	
GFDLNGGGVG	(SAP-I, Mw 892)
GFDLTGGGVG	(Thr ⁵]-SAP-I, Mw 879)
GFALGGGGVG	(Ala ³ , Gly ⁵]-SAP-I, Mw 791)
Suborder Echinina	
Family Strongylocentrotidae	
<i>Strongylocentrotus nudus</i>	
GFDLNGGGVG	(SAP-I, Mw 892)
GFSLSGGGVG	([Ser ^{3,5}]-SAP-I, Mw 837)
GFALGGGGVG	([Ala ³ , Gly ⁵]-SAP-I, Mw 791)
GFSLGGGGVG	([Ser ³ , Gly ⁵]-SAP-I, Mw 807)
GFDLTGGGVG	([Thr ⁵]-SAP-I, Mw 879)

(continued Table 1)

<i>Strongylocentrotus purpuratus</i>	
GFDLNGGGVG	(SAP-I, Mw 892)
GFALGGGGVG	([Ala ³ , Gly ⁵]-SAP-I, Mw 791)
GFSLTGGGGVG	([Ser ³ , Thr ⁵]-SAP-I, Mw 851)
<i>Hemicentrotus pulcherrimus</i>	
GFDLNGGGVG	(SAP-I, Mw 892)
GFDLTGGGGVG	([Thr ⁵]-SAP-I, Mw 879)
GFSLNGGGVS	([Ser ^{3,10}]-SAP-I, Mw 894)
SFALGGGGVG	([Ser ¹ , Ala ³ , Gly ³]-SAP-I, Mw 821)
GFSLSGSGVD	([Ser ^{3,5,7} , Asp ¹⁰]-SAP-I, Mw 925)
Family Echinometridae	
<i>Echinometra mathaei</i> (type A)	
GYLSGGGAVD	([Tyr ² , Ser ^{3,5} , Ala ⁸ , Asp ¹⁰]-SAP-I, Mw 925)
GFALSGGGVG	([Ala ³ , Ser ⁵]-SAP-I, Mw 821)
GFSLSGGGGVG	([Ser ^{3,5}]-SAP-I, Mw 837)
GFDLTGGGGVG	([Thr ⁵]-SAP-I, Mw 879)
<i>Echinometra mathaei</i> (type B)	
GYLSGGGAVD	([Tyr ² , Ser ^{3,5} , Ala ⁸ , Asp ¹⁰]-SAP-I, Mw 925)
GYNLNGDRID	([Try ² , Asn ³ , Asp ^{7,10} , Arg ⁸ , Ile ⁹]-SAP-I, Mw 1136)
GFSLSGGGGVG	([Ser ^{3,5}]-SAP-I, Mw 837)
GFDLTGGGGVG	([Thr ⁵]-SAP-I, Mw 879)
<i>Anthocidaris crassispina</i>	
GFDLTGGGGVG	([Thr ⁵]-SAP-I, Mw 879)
GFDLSGGGGVG	([Ser ⁵]-SAP-I, Mw 865)
GFSLSGSGVG	(Ser ^{3,5,7}]-SAP-I, 867)
<i>Heterocentrotus mammillatus</i>	
GTLP TGSGVS	([Thr ^{2,5} , Leu ³ , Pro ⁴ , Ser ^{7,10}]-SAP-I, Mw 875)
GFEMGGTGVG	([Glu ³ , Met ⁴ , Gly ⁵ , Thr ⁷]-SAP-I, Mw 911)
GYNLGGGGID	([Tyr ² , Asn ³ , Gly ⁵ , Ile ⁹ , Asp ¹⁰]-SAP-I, Mw 922)
GFGLSGGGIG	([Gly ³ , Ser ⁵ , Ile ⁹]-SAP-I, Mw 821)
Subclass Irregularia	
Order Clypeasteroida	
Suborder Clypeasterina	
Family Clypeasteroidae	
<i>Clypeaster japonicus</i>	
DSDSAQNLI	(SAP-III, Mw 1019)
GTDSAQNLI	([Gly ¹ , Thr ²]-SAP-III, Mw 975)
SDSAQNLI	(Des-Asp ¹ -SAP-III, Mw 904)
DSDSAHLI	(Des-Asn ⁷ -[His ⁶]-SAP-III, Mw 914)
DTDSAHLI	(Des-Asn ⁷ -[Thr ² , His ⁶]-SAP-III, Mw 928)
NTDSAHLI	(Des-Asn ⁷ -[Asn ¹ , Thr ² , His ⁶]-SAP-III, Mw 928)
GTDSAHLI	(Des-Asn ⁷ -[Gly ¹ , Thr ² , His ⁶]-SAP-III, Mw 870)
SDSAHLI	(Des-Asp ¹ , Asn ⁷ -[His ⁶]-SAP-III, Mw 799)
DSDSAFLI	(Des-Asn ⁷ -[Phe ⁶]-SAP-III, Mw 924)
Suborder Laganina	
Family Atriclepeidae	
<i>Atriclepeus manni</i>	
DSDSAHLI	(Des-Asn ⁷ -[His ⁶]-SAP-III, Mw 914)
DTDSAHLI	(Des-Asn ⁷ -[Thr ² , His ⁶]-SAP-III, Mw 928)
TDSAHLI	(Des-Asp ¹ , Asn ⁷ -[Thr ² , His ⁶]-SAP-III, Mw 813)

* Amino acids in the sequences are given a one-letter abbreviation: asparagine (N), aspartic acid (D), alanine (A), arginine (R), isoleucine (I), glycine (G), glutamine (Q), glutamic acid (E), cystine ($\bar{C}\bar{C}$), cysteine (C), serine (S), tyrosine (Y), tryptophan (W), valine (V), histidine (H), phenylalanine (F), proline (P), methionine (M), lysine (K) and leucine (L).

TABLE 2. Respiratory stimulating effect of sperm-activating peptides on sea urchin spermatozoa

	Spermatozoa used			
	<i>Diadema setosum</i>	<i>Glyptocidaris crenularis</i>	<i>Hemicentrotus pulcherrimus</i>	<i>Clypeaster japonicus</i>
SAP-IV	+	—	—	—
SAP-IIB	—	+	—	—
SAP-IIA	—	±	—	—
SAP-I	—	—	+	—
SAP-III	—	—	—	+

The respiratory-stimulating activity of a sperm-activating peptide is expressed as a plus sign (+) when the peptide stimulated sperm respiration one half-maximally less than 5 nM. When the half-maximal respiratory stimulation was induced by a peptide concentration between 5 and 500 nM, the plus-minus sign (±) was used. Practically no respiratory stimulation was depicted by a minus sign (—).

SPERM-ACTIVATING PEPTIDES

During the last ten years, my collaborators and I purified sixty-three sperm-activating peptides from the solubilized jelly layer obtained from eggs of fifteen sea urchin species distributed over four taxonomic orders (Table 1) [62, 67–75]. These peptides essentially demonstrate the same biological activity toward a given sea urchin spermatozoa although the biological activities of the peptides are specific at the ordinal level (Table 2) [76–77]. Considering structure and biological specificity, the peptides can be classified into four groups, i.e., sperm-activating peptide I from the species in the order Echinoidea, sperm-activating peptide II (subclass A and B) from the species in the order Arbacioida, sperm-activating peptide III from the species in the order Clypeasteroida and sperm-activating peptide IV from the species in the order Diadematoida. These groups of peptides may be abbreviated as SAP-I, SAP-II, SAP-III and SAP-IV [64].

The peptides stimulate the decreased respiration rates of sea urchin spermatozoa due to the acidification of sea water, back to the level of respiration rates in normal sea water [68]. The stimulated respiration rate does not exceed that of spermatozoa in normal sea water even if large amounts of peptides are added to the sperm suspension medium. The respiratory stimulation induced by the peptide continues for a few minutes and then sperm respiration rates usually decline to the basal

rate normally observed at that particular pH. After reaching the basal state, spermatozoa can again be stimulated by the addition of peptide. This is also true when respiratory stimulation is induced by solubilized jelly layer. When a limited concentration of peptide is used for respiratory stimulation of spermatozoa and the sperm suspending medium is centrifuged before the second addition of peptide, the resultant supernatant fluid, upon examination for respiration-stimulating activity toward spermatozoa, does not stimulate sperm respiration [78]. This suggests that the peptide binds tightly to the spermatozoa. Smith and Garbers reported that *Strongylocentrotus purpuratus* spermatozoa had approximately 6,000–8,000 binding sites (receptors)/cell specific for SAP-I [79]. We suggested that in *Hemicentrotus pulcherrimus* spermatozoa, the receptors exclusively localize on the sperm tail [78].

Monensin, an ionophore that catalyzes an electro-neutral Na^+/H^+ exchange across the cell membrane stimulates sea urchin sperm respiration and motility one half-maximally at about 1–10 μM , and induces a Na^+ -dependent net proton efflux as well as an increased flux of Na^+ in both directions across the cell membrane [61, 68, 80–82]. Sperm-activating peptide stimulate sperm respiration one half-maximally at about 10–100 pM. The respiratory stimulation induced by SAP-I or monensin is dependent on the concentration of external Na^+ [61, 68, 80, 82]. Approximately 50 mM Na^+ is required for half-maximal respiratory responses to

peptides or monensin. There seem to be similarities between the effects of SAP-I and monensin. It is well known that many types of metabolic activation in cells are induced by intracellular alkalization [83]. Thus, induction of respiratory stimulation by sperm-activating peptides may be explained by the hypothesis that the peptides trigger Na^+/H^+ exchange across sperm plasma membrane and raise the intracellular pH [43, 84].

It has been known that spermatozoa from various species including sea urchin spermatozoa possess enzymes such as adenylate cyclase, guanylate cyclase, cyclic nucleotide phosphodiesterase, cyclic nucleotide-dependent protein kinases and phosphoprotein phosphatases which are involved in cyclic nucleotide metabolism [84–97]. In many instances, these enzymes possess higher specific activity in spermatozoa than in other tissues.

Sperm-activating peptides cause transient increases in sea urchin sperm cGMP concentrations as well as cAMP concentrations in both acidic and normal sea water [63, 69, 73]. Half-maximal elevations of cGMP are 2×10^{-9} M and half-maximal elevations of cAMP are 2×10^{-8} M peptide (Fig. 2). The increases in cGMP concentrations are explained by transient activation of the membrane form of guanylate cyclase, which is a major protein of sperm tail plasma membrane [43, 88, 98–102].

Ward and Vacquier reported that within seconds after addition of solubilized jelly layer prepared from *Arbacia punctulata* to an *Arbacia punctulata* sperm suspension, a 160 kDa sperm protein disappeared and a new protein appeared at 150 kDa on an SDS-polyacrylamide gel [103–105]. The extent of the change was a function of jelly concentration but, at a given jelly concentration, was independent of incubation time. The change is specific for *Arbacia punctulata* jelly. Thereafter, we demonstrated that the factor responsible for the change is a sperm-activating peptide whose sequence is Cys-Val-Thr-Gly-Pro-Gly-Gly-Val-Gly-Gly-Gly-Arg-Leu-NH₂ (SAP-IIA) [69]. Similar changes are commonly observed in sea urchin spermatozoa of many species treated with a specific sperm-activating peptide [70–76]. *Hemicentrotus pulcherrimus* spermatozoa possess several major proteins and one of them, which was recently identified as guanylate cyclase, changes its relative mobility on SDS-polyacrylamide gels from 131 kDa to 128 kDa upon treatment with SAP-I (Fig. 3) [106]. The 160 kDa protein of *Arbacia punctulata* spermatozoa can be labeled with ³²P-orthophosphate when intact *Arbacia punctulata* spermatozoa are incubated in sea water containing ³²P-orthophosphate. The label which is in the form [³²P]-phosphoserine, disappears completely after exposure by the spermatozoa to the solubilized

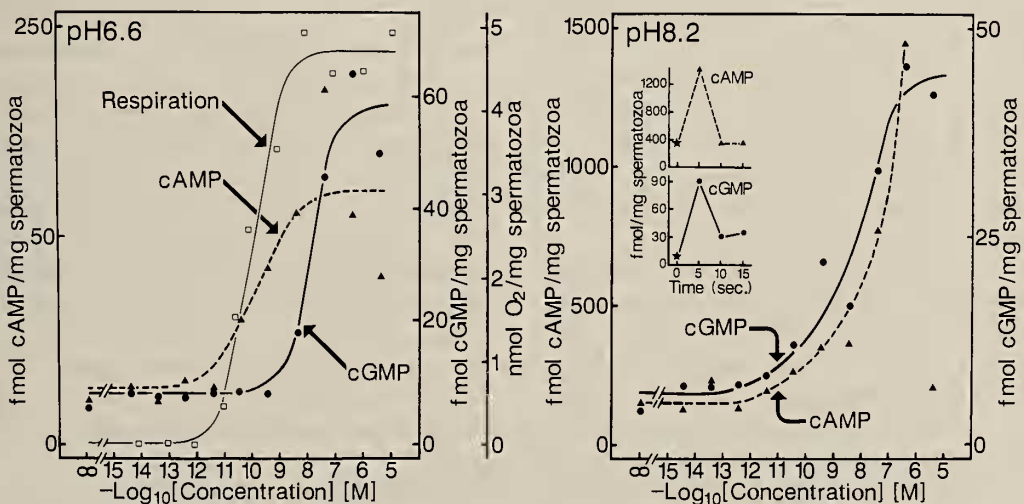


FIG. 2. Concentration-dependent effect of SAP-I on cAMP and cGMP concentrations and respiration of *Hemicentrotus pulcherrimus* spermatozoa. See [ref. 73] for experimental details.

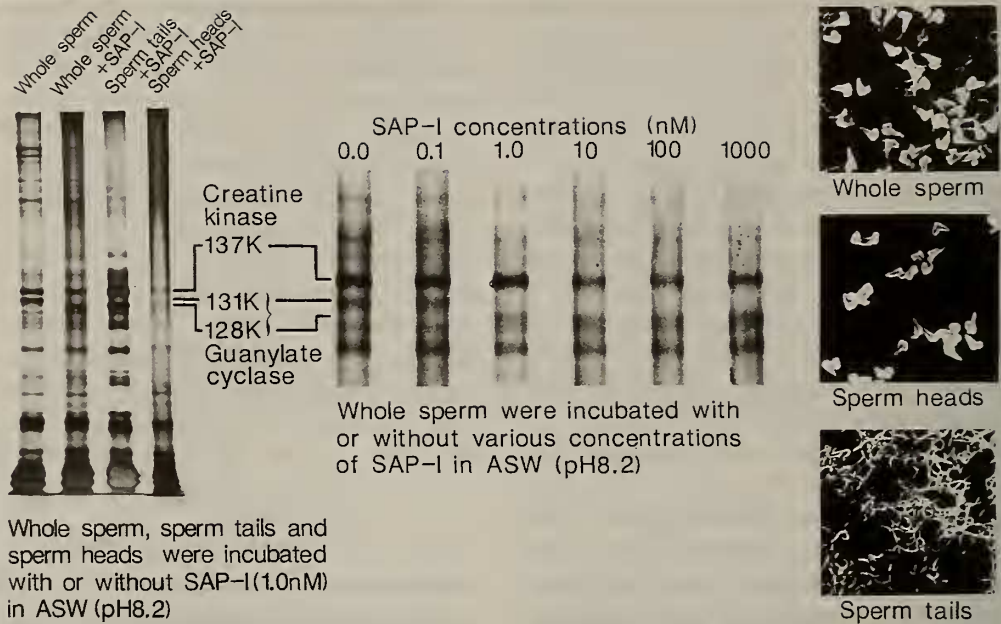


Fig. 3. Effect of SAP-I on the electrophoretic mobility of a high molecular weight protein of *Hemicentrotus pulcherrimus* spermatozoa. See [ref. 78] for experimental details.

jelly layer of *Arbacia punctulata* or SAP-IIA, without the corresponding appearance of radioactivity at 150 kDa [103, 107–108]. The 160 kDa and 150 kDa proteins have proven to be two forms of the same protein and identified as guanylate cyclase [104]. Loss of phosphate from guanylate cyclase and change in the molecular form which is dependent upon external Na^+ and Ca^{2+} concentrations result in a large decrease in the specific activity of the enzyme [95–96]. Therefore, sperm-activating peptides appear to cause an initial transient activation and subsequent inactivation of guanylate cyclase [102]. The mechanism in detail, however, remains to be elucidated.

Guanylate cyclase of *Arbacia punctulata* spermatozoa has been proven to be the receptor for SAP-IIA but not for SAP-I [98–101, 109]. Dangott and Garbers reported that the receptor for SAP-I on *Strongylocentrotus purpuratus* spermatozoa is a 77 kDa protein which has no known enzyme activity [110–112]. Receptors for SAP-IIB, SAP-III and SAP-IV have yet to be identified.

FUCOSE SULFATE GLYCOCONJUGATE

It has long been believed that a fucose sulfate polysaccharide or a fucose-sulfate-rich complex in the jelly layer of sea urchins is the only substance responsible for the induction of the acrosome reaction in sea urchin spermatozoa [9, 11–16]. Recently, my collaborators and I demonstrated that full induction of the acrosome reaction in *Hemicentrotus pulcherrimus* spermatozoa with a fucose sulfate glycoconjugate requires a specific co-factor, i.e., sperm-activating peptide I (Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly) [17–18, 65]. The acrosome reaction of sea urchin spermatozoa is accompanied by Na^+ and Ca^{2+} influx, and H^+ and K^+ efflux [44, 113–128]. Activation of sea urchin spermatozoa by sperm-activating peptides is also accompanied by these ionic changes which cause an increased intracellular pH and trigger sperm plasma membrane depolarization [82, 129–130].

In 1952, Dan first discovered using sea urchin gametes that the acrosome reaction is essential for fertilization and that the factor responsible for the

acrosome reaction exists in an extracellular matrix called the jelly layer [1]. Since then many investigators have made tremendous efforts to identify this factor and to clarify the mechanism of the reaction [2–16]. Ishihara and Dan reported that treatment of the solubilized jelly layer of *Hemicentrotus pulcherrimus* with trypsin and pronase results in a reduction of the acrosome reaction-inducing ability to about half of that with the untreated jelly [5]. SeGall and Lennarz isolated a fucose sulfate-containing substance containing less than 30% protein from the solubilized jelly layer of four species of sea urchin by gel filtration and ion-exchange chromatography [13]. They demonstrated that the acrosome reaction-inducing capacity of the substance resides solely in the fucose sulfate polysaccharide, although the inducing activity decreases during purification. They concluded that this decrease in activity is due to the loss of Ca^{2+} during purification. Garbers *et al.* reported that a fucose-sulfate-rich complex purified from the solubilized jelly layer of *Strongylocentrotus purpuratus* is capable of elevating sperm cAMP concentrations and inducing the

acrosome reaction [16]. They also demonstrated that NaOH or pronase treatment of the complex results in significant reductions in both acrosome reaction-inducing potency and maximal ability of the complexes to elevate cAMP concentrations.

The fucose sulfate glycoconjugate purified from the solubilized jelly layer of *Hemicentrotus pulcherrimus* induces the acrosome reaction in *Hemicentrotus pulcherrimus* spermatozoa, but the potency is about half that of the unfractionated jelly (Table 3) [18, 65]. When a sperm-activating peptide-free macromolecular fraction is prepared from the solubilized jelly layer of *Hemicentrotus pulcherrimus* which is composed of a sialoglycoprotein and a fucose sulfate glycoconjugate, the rates of the acrosome reaction induced by the fraction are also about half that of the original unfractionated jelly [17]. The addition of synthetic SAP-I to the fraction or the fucose sulfate glycoconjugate increases the rate of the acrosome reaction to a level comparable with that of the unfractionated jelly at all pHs tested. SAP-I promotes the acrosome reaction with fucose sulfate glycoconjugate in *Hemicentrotus pulcherrimus* sperma-

TABLE 3. The rate of acrosome reaction in *Hemicentrotus pulcherrimus* spermatozoa in the presence or absence of SAP-I treated with crude jelly, sialoglycoprotein, FSG, pronase-digested FSG and carboxymethylated FSG

	Percentage of Reacting Spermatozoa	
	Exp. 1	Exp. 2
Crude jelly	90%	87%
FSG + none	32	45
+ SAP-I	75	81
Pronase-digested FSG		
+ none	15	
+ SAP-I	49	
Carboxymethylated FSG		
+ none		24
+ SAP-I		54
Sialoglycoprotein		
+ none		3
+ SAP-I		5
ASW alone	3	2

Spermatozoa were incubated in 0.5 ml of ASW or ASW containing crude jelly [62.5 (Exp. 1) or 100 (Exp. 2) nmol fucose/ml], sialoglycoprotein (100 nmol sialic acid/ml), fucose sulfate glycoconjugate (FSG) [62.5 (Exp. 1) or 100 (Exp. 2) nmol fucose/ml], pronase-digested FSG (62.5 nmol fucose/ml), and carboxymethylated FSG (100 nmol fucose/ml) with or without SAP-I (0.5 μM).

tozoa in a concentration-dependent manner with a half-maximal rate of 4×10^{-10} M which is almost the same concentration of peptide needed to cause half-maximal stimulation of sperm respiration [18]. Since SAP-I alone does not induce the acrosome reaction and sialoglycoprotein, another large molecular weight component of the jelly layer, also does not show any appreciable potency for induction of the acrosome reaction, we think that

the major substance responsible for induction of the acrosome reaction is the fucose sulfate glycoconjugate and SAP-I promotes the induction of the acrosome reaction by acting as a co-factor [65].

The fucose sulfate glycoconjugate isolated from the solubilized jelly layer of *Hemicentrotus pulcherrimus* contains a large amount of protein [65]. The proteins associated with fucose sulfate can not be removed even when the glycoconjugate is dis-

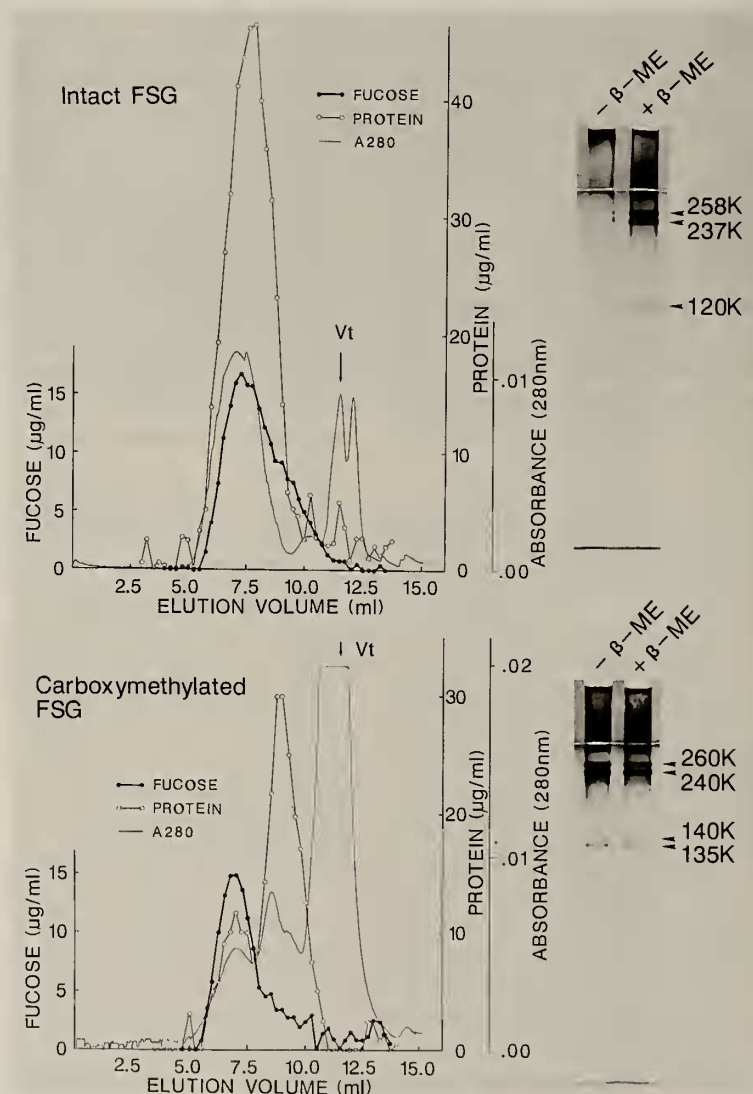


FIG. 4. HPLC and SDS-PAGE of intact and carboxymethylated fucose sulfate glycoconjugate isolated from the solubilized jelly layer of *Hemicentrotus pulcherrimus*. Fucose sulfate glycoconjugate is abbreviated as FSG in the figure. See [ref. 65] for experimental details.

solved in 7 M urea or 0.1% SDS and chromatographed on a Sepharose CL-2B or a TSK G-6000 PW column (Fig. 4). Furthermore, no protein band is detectable on the electrophoretogram of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the intact fucose sulfate glycoconjugate without a reducing agent such as 2-mercaptoethanol. These results suggest that the protein may be covalently bound to the polysaccharide portions of the glycoconjugate. When the glycoconjugate is dissolved in an SDS solution containing 2-mercaptoethanol and subjected to SDS-PAGE in the presence of 2-mercaptoethanol, two major (258 kDa and 238 kDa) and one minor (120 kDa) protein bands appeared. A reduced and carboxymethylated fucose sulfate glycoconjugate (CmFSG) separated into two major (260 kDa and 240 kDa) and two minor (140 kDa and 135 kDa) protein bands on an SDS-gel despite the absence of 2-mercaptoethanol (Fig. 4). When CmFSG was subjected to HPLC on a TSK G-6000 PW column, a fucose-containing material, which possesses about 30% of the protein originally associated with the fucose sulfate glycoconjugate, was separated from the rest of the proteins. These proteins separated into 260 kDa, 240 kDa, 140 kDa and 135 kDa components by SDS-PAGE. This indicates that the fucose sulfate glycoconjugate consists of fucose sulfate polymers bound to a core protein and several other protein components associated with each other through disulfide bounds. In order to confirm the presence of disulfide bonds in the fucose sulfate glycoconjugate, free sulfhydryl groups in the fucose sulfate glycoconjugate are first treated with non-radioactive iodoacetic acid, which results in the carboxymethylation of about half of the cysteine residues present in the original glycoconjugate, and they are then reduced and carboxymethylated with [^{14}C] iodoacetic acid. The resulting labeled carboxymethylated glycoconjugate was analyzed by SDS-PAGE, and showed that protein bands corresponding to 140 kDa and 135 kDa proteins have strong radioactivity although weaker radioactivity was detected in the 260 kDa, 240 kDa, 110 kDa and 87 kDa protein bands. In amino acid analyses of protein components isolated from CmFSG, a 140 kDa and 135 kDa protein fraction contained the largest mol%

of carboxymethylated cysteine. These results suggest that 140 kDa and 135 kDa proteins, which are probably the same protein(s) detected in a 120 kDa protein band in SDS-PAGE of the intact fucose sulfate glycoconjugate with 2-mercaptoethanol have more sulfhydryl groups than other protein components available for disulfide bond formation and may serve to make bridge between the polysaccharide bound core protein and other protein components.

Complete carboxymethylation of cysteine residues in the major acrosome reaction-inducing jelly molecules, the fucose sulfate glycoconjugate, results in the release of most of the constituent proteins from the glycoconjugate and about a 50% decrease in the acrosome reaction-inducing capacity of the glycoconjugate [65]. When about 70% of the constituent proteins is removed from the fucose sulfate glycoconjugate by pronase digestion, the resulting fucose-rich glycoconjugate loses more than 50% of the acrosome reaction-inducing activity of the original untreated glycoconjugate (Table 3) [18]. In both cases, the decreased potency could be partially restored by the addition of SAP-I, monensin, 3-isobutyl-1-methylxanthine (IBMX) which is an inhibitor for cyclic nucleotide phosphodiesterase or a Ca^{2+} -ionophore, A23187 [18]. Fucoidan, a polymer of L-fucose-4-sulfate which is a major sugar component in the fucan sulfate isolated from the solubilized jelly layer of *Hemicentrotus pulcherrimus*, does not appreciably induce the acrosome reaction of *Hemicentrotus pulcherrimus* spermatozoa [65]. These results suggest that the protein moiety of fucose sulfate glycoconjugate is involved in the acrosome reaction although the mechanism remains unclear.

FUTURE PROSPECTS

It is known that the acrosome reaction is dependent on increased intracellular pH [29–30]. SAP-I increases an intracellular Ca^{2+} which is coupled to Na^+ entry and induces a rise in intracellular pH in *Strongylocentrotus purpuratus* spermatozoa [129–130]. Thus, we may presume that SAP-I promotes an acrosome reaction with fucose sulfate glycoconjugate by increasing intracellular pH in sperm cells.

A fucose-sulfate-rich complex obtained from the solubilized jelly layer of *Strongylocentrotus purpuratus* causes Ca^{2+} -accumulation, elevates cAMP and induces the acrosome reaction [16, 44, 114, 131-134]. In connection with this, it should be noted that monoclonal antibodies against a 210 kDa glycoprotein purified from *Strongylocentrotus purpuratus* sperm plasma membrane cause an increase in intracellular Ca^{2+} of *Strongylocentrotus purpuratus* spermatozoa and induce the acrosome reaction when intracellular pH is increased with NH_4Cl [118, 135-139]. Antibodies induce the cAMP-dependent phosphorylation of sperm histone H1 as occurs upon treatment of spermatozoa with the solubilized jelly layer of *Strongylocentrotus purpuratus* [91-93]. Similar results are presented by Sendai *et al.* using sea urchin gametes of *Strongylocentrotus intermedius* [38-39]. However, an ionophore A23187 which is known to induce the acrosome reaction and acts as a co-factor of a pronase-digested fucose sulfate glycoconjugate for induction of the acrosome reaction neither induces histone H1 phosphorylation nor increases cAMP concentrations in sea urchin spermatozoa [9, 18, 92, 140]. This supports the idea which suggests that cAMP is not directly involved in the acrosome reaction. Since a fucose-sulfate-rich complex obtained from *Strongylocentrotus purpuratus* jelly induces the elevation of inositol trisphosphate and the elevation is dependent on external Ca^{2+} , the products generated from stimulated PI turnover in sea urchin spermatozoa may be potential second messengers in induction of the acrosome reaction [141].

Sea urchin gametes have long been used for studying fertilization and development [3, 29, 43, 64, 84, 127, 142-152]. It is not an exaggeration to say that sea urchin are one of the most extensively used experimental animals in basic biology. However, we must realize that we do not know much about the substances involved in sea urchin gamete interaction. An understanding of this would not only lead to an understanding of fertilization in mammals and other animals, but it would serve as a valuable indicator of mechanisms which can be anticipated in cells other than gametes. Sperm-activating peptides cause many biochemical responses in sea urchin spermatozoa

such as increases in cGMP and cAMP concentrations, induction of ionic exchanges across the plasma membrane and increases in intracellular pH. The mechanism of action of the peptides resembles in many aspects that of the atrial natriuretic peptides, although the mechanism has yet to be described in detail [153-156]. Since de Bold *et al.* suggested that a factor released from the atria of the heart evokes a variety of physiological responses affecting cardiovascular homeostasis, intensive studies have been carried out in an attempt to identify the factor and a receptor(s) for the factor [153-159]. Recently, the structure of a receptor for atrial natriuretic peptides was determined by Chinkers *et al.* as a result of advanced research on a sperm-activating peptide receptor [160]. This serves as an excellent example for demonstrating that the results of research in one field can be applied to solving the problems in other fields.

The receptor for the fucose sulfate glycoconjugate, which is the major substance responsible for induction of the acrosome reaction has yet to be isolated. Study of the receptor protein appears to have great significance because questions have arisen as to whether or not this protein could also represent a calcium channel. Parallel to the isolation and characterization of the receptor, the function of primary and secondary messengers will be an important future area of research, much as in other cells.

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